AD_____

Award Number: DAMD17-99-1-9219

TITLE: An Estrogen Receptor-Selective Coactivator: A Potential Regulator of Tamoxifen Effectiveness in Breast Cancer Treatment and Prevention

PRINCIPAL INVESTIGATOR: Ramji R. Rajendran

CONTRACTING ORGANIZATION: University of Illinois
Champaign, Illinois 61820-6242

REPORT DATE: September 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2001	Annual Summary (1 Sep 00 - 31 Aug 01)

4. TITLE AND SUBTITLE
An Estrogen Receptor-Selective Coactivator: A Potential Regulator of Tamoxifen Effectiveness in Breast Cancer Treatment and Prevention

5. FUNDING NUMBERS
DAMD17-99-1-9219

6. AUTHOR(S)
Ramji R. Rajendran

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Illinois
Champaign, Illinois 61820-6242

E-Mail: rajendra@students.uiuc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Much evidence demonstrates that the estrogen receptor (ER) is involved in breast cancer development and progression. We have isolated a novel 97 kDa DEAD box RNA helicase protein (p97), from breast cancer cells, which interacts with the estrogen receptor E and F domains in the presence of either estradiol or trans-hydroxytamoxifen. We have further confirmed the interaction of p97 with ER using both GST-pulldown analysis and a mammalian two-hybrid assay. We have localized a C-terminal (662-864) portion of p97 that specifically interacts with the EF region of the estrogen receptor. In reporter gene assays of transiently transfected cells, p97 decreases the transcriptional activity of agonist-occupied estrogen receptor, progesterone receptor, glucocorticoid receptor, and retinoic acid receptor. However, p97 has no effect on the transcriptional activity of p53 or VP16. p97 has intrinsic repression activity on a constitutively active promoter when recruited to it with a Gal4 DNA binding domain. We have identified a small region of p97 that retains this intrinsic repression activity. Trichostatin A, a selective histone deacetylase inhibitor, is able to reverse the repressive activity of p97 on ER, suggesting an involvement of the histone deacetylase complex with p97. Further analysis of this coregulator will allow insight into nuclear receptor function and its role in breast cancer.

14. SUBJECT TERMS
Breast Cancer, Estrogen Receptor, Transcriptional Coregulator, Estrogen, Tamoxifen

15. NUMBER OF PAGES
15

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction:

The estrogen receptor (ER) is a ligand inducible transcriptional activator that has been implicated in both the diagnosis and treatment of breast cancer. Co-regulator proteins of the estrogen receptor can selectively interact with and mediate the effect of antagonist versus agonist occupied receptors [1, 2]. When the balance of co-regulators is shifted, the receptor pharmacology can be changed [5]. For example, a tamoxifen occupied ER can become estrogen-like and stimulatory to cells when there is a loss of ER co-repression or a gain in ER co-activation [7]. To identify factors that are involved in the activity of the estrogen receptor, we previously performed a yeast two-hybrid screen using the ERα E and F domains [3]. One such factor isolated in this screen interacts with the estrogen receptor in a ligand dependent manner. This protein is a novel DEAD box RNA helicase of 97 kDa (p97.) We have examined the role of p97 interaction with the estrogen receptor and its ability to affect the transactivation of nuclear receptors. We have focused on understanding the role of this protein as a coregulator and dissecting regions of p97 involved in its helicase activity, interaction with the estrogen receptor, and transcriptional repression. We have also determined that p97 is localized in the nucleus of cells and is concentrated at the nucleoli. We aim to better understand the activity of this novel coregulator protein and determine its role in estrogen receptor-positive breast cancer.

Body:

Transcriptional co-regulator proteins are known to be involved in the modulation of transcriptional activity of the estrogen receptor. We have previously cloned and characterized a novel 97 kDa DEAD box RNA helicase (p97) that acts as a nuclear receptor coregulator. We have also shown that this protein is able to interact with and repress the transcriptional activity of nuclear receptors. In the past year, we have further characterized the biochemical activity of p97 as a RNA helicase. We have also defined a region of interaction of p97 with nuclear receptors. Furthermore, we have made truncations of the p97 protein that show that the ATP dependent RNA helicase activity of p97 is separable from the transcriptional repression activity. p97 is also able to repress constitutively active reporters that it is recruited to, and a particular region of p97 is also able to maintain this intrinsic repression activity. We have shown that transcriptional repression by the p97 protein is relieved by treatment with the histone deacetylase inhibitor Trichostatin A. We have also shown that p97 localizes to the nuclei of cells with a substantial concentration at the nucleoli. We aim to further examine the role of histone deacetylase proteins in the transcriptional repression of nuclear receptors mediated by p97. Further understanding of the activity of this novel protein will enable us to better understand the role of nuclear receptors as transcriptional regulators in breast cancer.

After determining that p97 has the sequence motifs to be classified as a DEAD box RNA helicase, we characterized the biochemical helicase activity of p97 by performing RNA dependent ATPase assays. We determined ATP dependent RNA unwinding by measuring the amount of hydrolysis of ATP to AMP by a purified GST-p97(33-662) protein in the presence of increasing amounts of MCF-7 breast cancer cell total RNA. Poly U RNA did not cause any change in the amount of hydrolysis of ATP by p97 at any concentration. This suggests that particular RNA molecules from MCF-7 total RNA are necessary as a substrate for p97. We also found that prior treatment of MCF-7 total RNA with RNase prevents unwinding of RNA by p97 as measured by hydrolysis of ATP. Thus, the ATPase activity is dependent on the presence of RNA. These ATPase experiments show that p97 can biochemically be characterized as an ATP dependent RNA helicase, as suggested by the phylogenetic motifs present in its sequence.

To characterize the interaction of p97 with the estrogen receptor, we performed *in vitro* an *in vivo* interaction assays. The wildtype ERα interacts with the full-length p97(as a GST-fusion protein) in the presence of trans-hydroxy tamoxifen (TOT) or estradiol (E2), but not in the absence of these receptor ligands. Next, we aimed to determine the region of interaction between the estrogen receptor and the p97 protein. To do this, we created GST fusion proteins of p97(1-865), p97(1-656), p97(413-664), and p97(663-865). GST pull-down experiments revealed that only the full-length p97 and the p97(663-865) interact with the estrogen receptor. We then examined which region of ERα is

able to interact with p97(657-865) using *in vitro* translated products of ERα-(ABC), ERα-(DEF), and ERα-(EF). p97(657-865) does not interact with ERα-(ABC), but does interact with ERα-(DEF) and ERα-(EF). The receptor interaction was strongest in the presence of TOT, rather than E2 or unliganded. This result is consistent with the original yeast two-hybrid screen because the ERα-(EF) bait used interacted with the C-terminus of p97 with increased affinity in the presence of TOT. In addition, the F domain of ERα has been shown to be important for antiestrogen ligand modulation by the estrogen receptor[4]. ERβ, and the ERβ-(DEF) regions also interact with GST-p97(657-865) in the presence of either E2 or TOT. The DEF regions of the progesterone receptor b subtype (PRb) and the glucocorticoid receptor (GR) also interact with p97(657-865) in the presence of agonist and antagonist, but not in the absence of ligand.

The *in vitro* interaction between p97 and ERα was confirmed in a cellular context using a mammalian two-hybrid system. p97 was fused to the GAL4 DNA binding domain (DBD) and ERα was fused to the VP16 activation domain (AD). The two proteins interact, thereby activating the reporter construct in the presence of ER and TOT, but not in the absence of ligands.

Using transient transfections and reporter gene assays, we found that p97 represses the magnitude of transcriptional activity of ERα and ERβ, PR b, GR, and retinoic acid receptor α (RARα) by 60%-90% of the maximal activity without added p97. However, p97 is unable to repress the transcriptional activity of p53 or the VP16 viral protein transcriptional activator. We next analyzed the ability of truncated regions of p97 to repress the activity of ERα. pCMVFLAGp97(1-412), which contains all of the DEAD box motifs, did not affect the transcriptional activity of the estrogen receptor. However, pCMVFLAGp97(413-865) represses ERα slightly better than the full-length p97. Since p97(657-865) interacts with ERα, we divided the pCMVFLAGp97(413-865) into two portions. Neither pCMVFLAGp97(413-663) nor pCMVFLAGp97(664-865) can repress ERα on their own. Thus, both the region of p97 that interacts with ERα and the region of p97 from 413-663 are critical for transcriptional repression of ERα.

To determine if p97 may itself have the ability to directly repress gene promoter activity, we used a constitutively active reporter gene, and a p97 construct in which the Gal4 DNA binding domain was fused to the amino terminus of full length p97. This Gal4-p97 protein was able to repress a constitutively active SV40 promoter with five Gal4 binding sites upstream of a luciferase reporter gene. Thus, p97 has the ability to repress promoters by recruitment alone, and is not dependent on other factors recruited by ER or other nuclear receptors. We have also found that the Gal4 DNA binding domain fusion of p97(413-663) also has intrinsic repression activity, so this small region if p97 acts as a repression domain. In many cases, histone acetyltransferase enzymes enhance transcriptional activity, and histone deacetylases repress transcription. Trichostatin A, a drug that inactivates histone deacetylases[6], relieves the repressive action of p97 on ER transcriptional activity. Therefore, p97 may recruit histone deacetylase proteins.

We generated a polyclonal antibody against a peptide present in the p97 sequence. In Western analysis, this antibody recognizes a 97 kDa band in MCF-7 breast cancer cell extracts. We used this antibody and the anti-ERα rat monoclonal antibody H222 to determine the localization of both p97 and ERα in untransfected MCF-7 cells and in CHO cells that have been transfected with both pCMV-p97 and pCMV-ERα. In both cases, p97 was found only in the nucleus with a significant amount of p97 concentrated at the nucleoli and some in the nucleolasm. It will be interesting to examine whether the fraction of p97 that is localized to the nucleoplasm is able to cause its repressive action or whether p97 is able to draw nuclear receptors to the nucleoli and sequester them from DNA response elements.

p97 is a novel coregulator of nuclear receptors. Further analysis of p97 as a nuclear receptor coregulator will lead to a better understanding of the role of this protein in nuclear receptor activity. Better insight into the activity of p97 and its interaction with other transcriptional regulatory proteins such as histone deacetylase complexes will provide a clearer picture to the role of p97 and its effects on estrogen receptor activity within breast cancer cells.

Key Accomplishments for 9/2000 to 9/2001

- Performed ATP hydrolysis assays with p97 protein to characterize its ATP dependent RNA helicase activity
- Identified that the EF region of the estrogen receptor interacts with p97(664-865) in a ligand dependent manner
- Determined that the N-terminal DEAD box motifs-containing region of p97 is not required for the repression of the estrogen receptor
- Determined that p97 is able to work as an intrinsic repressor on constitutively active promoters and that a region of p97(amino acids 413-663) is able to maintain this intrinsic repression activity
- Linked the ability of p97 to repress transcriptional activity of the estrogen receptor to histone deacetylase activity

Reportable Outcomes for Sept. 1, 2000 – Aug. 31, 2001

Manuscripts:

Nye, A.C., **Rajendran, R.R.**, Belmont, A.S.. (2001) The cell. Encyclopedia of the Human Genome; Nature Publishing Company in press.

Nye, A.C., **Rajendran, R.R.**, Katzenellenbogen, B.S., Belmont, A.S. (2001) Movie:The estrogen receptor alters large-scale chromatin structure. Trends in Cell Biology GFP in Motion CD-ROM, volume 2, ed. B Ludin and A Matus.

Abstract and Presentation:

Rajendran, R.R., Nye, A.C., Martini, P.G., Katzenellenbogen, B.S. "p97: A novel DEAD box RNA helicase that acts as a nuclear receptor coregulator." Poster presented at 83rd Annual Meeting of the Endocrine Society. Denver, Colorado, June 2001.

Award:

2001 Endocrine Society Travel Grant Award Recipient

Conclusions:

We are on track to complete the goals in our statement of work. We have determined that the full-length p97 sequence is able to repress the activity of nuclear receptors tested, but not the transcriptional activators VP16 or p53. We have been able to map the interaction domains between the ER and p97. We have found that p97 has the motifs to be characterized as an ATP dependent DEAD box RNA helicase and characterized this activity biochemically, although the N-terminal DEAD box motifs of p97 are dispensable for the repression of the estrogen receptor. We have shown that p97 is able to work as an intrinsic repressor on constitutively active promoters and identified a region of p97 that maintains this intrinsic repression activity. We have also generated and characterized an antibody to p97 and used this reagent to show that p97 is localized in the nucleus of cells. Furthermore, we have linked p97 ability to repress transcriptional activity of the estrogen receptor to histone deacetylase activity. We are now poised to perform studies to further elucidate the role of histone deacetylase proteins in the transcriptional repression activity of p97. We hope that a better understanding of the role of p97 in estrogen receptor transcriptional activity will lead to new approaches for the diagnosis and treatment of endocrine responsive breast cancer.

References:

[1] C. K. Glass, D. W. Rose and M. G. Rosenfeld, *Nuclear receptor coactivators*, Curr Opin Cell Biol, 9 (1997), pp. 222-32.

[2] N. J. McKenna, R. B. Lanz and B. W. O'Malley, *Nuclear receptor coregulators: cellular and molecular biology*, Endocr Rev, 20 (1999), pp. 321-44.

[3] M. M. Montano, K. Ekena, R. Delage-Mourroux, W. Chang, P. Martini and B. S. Katzenellenbogen, *An estrogen receptor-selective co-regulator that potentiates the effectiveness of antiestrogens and represses the activity of estrogens*, Proc Natl Acad Sci U S A, 96 (1999), pp. 6947-52.

[4] M. M. Montano, V. Muller, A. Trobaugh and B. S. Katzenellenbogen, *The carboxy-terminal F domain of the human estrogen receptor: role in the transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists*, Mol Endocrinol, 9 (1995), pp. 814-25.

[5] G. S. Takimoto, J. D. Graham, T. A. Jackson, L. Tung, R. L. Powell, L. D. Horwitz and K. B. Horwitz, *Tamoxifen resistant breast cancer: coregulators determine the direction of transcription by antagonist-occupied steroid receptors*, J Steroid Biochem Mol Biol, 69 (1999), pp. 45-50.

[6] J. Taunton, C. A. Hassig and S. L. Schreiber, *A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p*, Science, 272 (1996), pp. 408-11.

[7] X. Zhang, M. Jeyakumar, S. Petukhov and M. K. Bagchi, *A nuclear receptor corepressor modulates transcriptional activity of antagonist-occupied steroid hormone receptor*, Mol Endocrinol, 12 (1998), pp. 513-24.

Appendix 1: Abstract and Presentation:

Rajendran, R.R., Nye, A.C., Martini, P.G., Katzenellenbogen, B.S. "p97: A novel DEAD box RNA helicase that acts as a nuclear receptor co-regulator." Poster presented at 83rd Annual Meeting of the Endocrine Society. Denver, Colorado, June 2001.

P3-614 ★

P97: A NOVEL DEAD BOX RNA HELICASE THAT ACTS AS A NUCLEAR RECEPTOR COREGULATOR

RR Rajendran, AC Nye, PG Martini, BS Katzenellenbogen. *Cell and Structural Biology and Molecular and Integrative Physiology, University of Illinois and College of Medicine, Urbana, Illinois*

Using an MCF-7 breast cancer cell cDNA library in a yeast two-hybrid screen, we have isolated a novel gene encoding a protein that interacts with the estrogen receptor (ER) in the presence of either estradiol or trans-hydroxytamoxifen. The 864 amino acid protein has a predicted size of 97 kDa (p97). Its interaction with ER has been further confirmed using GST-pulldown and mammalian two-hybrid assays. The C-terminal (662-864) portion of p97 specifically interacts with the E region of the ER and does not interact with receptor domains ABC. The N-terminal part of p97 contains the conserved motifs present in ATP-dependent DEAD box RNA helicase proteins. We have characterized p97 helicase activity by demonstrating specific ATP hydrolysis by p97 in a dose dependent manner in the presence of MCF-7 total RNA. A GFP-p97 chimera is localized predominantly to the nucleolus, the intracellular location demonstrated for some other DEAD box RNA helicases. p97 mRNA is present at differing levels in all cell lines and tissues examined. In reporter gene assays of transiently transfected cells, p97 decreases the magnitude of estradiol-occupied ER transcriptional activity by up to 90%. It also represses the activity of the progesterone receptor, glucocorticoid receptor, and retinoic acid receptor, but it has no effect on the transcriptional activity of VP16. p97 has intrinsic repression activity on a constitutively active SV40 promoter when recruited to it with a Gal4 DNA binding domain. In order to understand the mechanism by which p97 works as a repressor, we used Trichostatin A (TSA), a selective histone deacetylase inhibitor. Intriguingly, we found that TSA is able to reverse the repressive activity of p97 on ER, suggesting an involvement of the histone deacetylase complex with p97. Further analysis of this unique coregulator should allow for insight into nuclear receptor function.

Appendix 2:

Curriculum Vitae
Ramji R. Rajendran

School	**Degree**	**Dates**	**Department**
University of Illinois, Urbana-Champaign, IL	Graduate Student	9/96-present	Cell and Structural Biology
University of Virginia, Charlottesville, VA	B.A.	8/92-5/96	Biology

Professional Experience

6/96-Present: Doctoral Student, Department of Cell and Structural Biology, University of Illinois at Urbana-Champaign, IL with Dr. Benita S. Katzenellenbogen

6/92-6/96: Undergraduate Research, Department of Opthalmology, University of Virginia, Charlottesville, VA with Dr. Federico Gonzalez-Fernandez

Honors and Awards

Endocrine Society Travel Grant Award Recipient (2001)

Department of Defense Breast Cancer Predoctoral Traineeship (1999-present)

Bioactoustics and Bioengineering in Radiation Oncology Training Program (1998-present)

Howard Hughes Undergraduate Research Fellowship (1993-1995)

Publications

Rajendran, R.R., Nye, A.C., Balsara, R.D., Martini, P.G.V., Belmont, A.S., and Katzenellenbogen, B.S. (2001) p97: a novel DEAD box RNA helicase and nuclear receptor co-regulator. in preparation.

Nye, A.C., **Rajendran, R.R.**, Belmont, A.S. (2001) The cell. Encyclopedia of the Human Genome; Nature Publishing Company in press.

Nye, A.C., **Rajendran, R.R.**, Katzenellenbogen, B.S., Belmont, A.S. (2001) Movie:The estrogen receptor alters large-scale chromatin structure. Trends in Cell Biology GFP in Motion CD-ROM, volume 2, ed. B Ludin and A Matus.

Nye, A.C., **Rajendran, R.R.**, Stenoien, D.L., Mancini, M.A., Katzenellenbogen, B.S., and Belmont, A.S. (2001) The estrogen receptor alters large scale chromatin structure. Submitted.

Sun, J., Meyers, M.J., Fink, B. E., **Rajendran, R.R.**, Katzenellenbogen, J.A., Katzenellenbogen, B.S. (1999) Novel ligands that funtion as selective estrogens or antiestrogens for estrogen receptor-α or estrogen receptor-β. Endocrinology, 140:800-804.

Rajendran, R.R., Van Niel, E.E., Stenkamp D.K., Cunningham L.L., Raymond P.A., Gonzalez-Fernandez F. (1996) Zebrafish interphotoreceptor retinoid-binding protein: Differential circadian expression among cone subtypes. J. Exp. Biol. 199, 2775-2787.

Wagenhorst B.B., **Rajendran, R.R.**, Van Niel, E.E., Bukelman A., Gonzalez-Fernandez F. (1995) Goldfish cones secrete a two-repeat interphotoreceptor retinoid binding protein. J. Mol. Evol. 41(6):646-656.

Abstracts:

Rajendran, R.R.,, Nye, A.C., Martini, P.G., Katzenellenbogen, B.S. (2001) p97: A novel DEAD box RNA helicase that acts as a nuclear receptor coregulator. Poster presented at 83rd Annual Meeting of the Endocrine Society. Denver, Colorado, June 2001.

Nye, A.C., Stenoien, D.L., **Rajendran, R.R.**, Katzenellenbogen, B.S., Mancini, M.A., Belmont, A.S. (2000) The estrogen receptor alters large scale chromatin structure. Dynamic Organization of Nuclear Function. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Nye, A.C., **Rajendran, R.R.**, Katzenellenbogen, B.S., Belmont, A.S. (1999) The estrogen receptor induces decondensation of large scale chromatin structure. Chromatin Structure and DNA function: Twenty-Five Years of the Nucleosome.

Rajendran, R.R., Stenkamp D.K., Raymond P.A., Van Niel, E.E, Cunningham L.L., Gonzalez-Fernandez F (1996) Species differences in interphotoreceptor retinoid Binding Protein (IRBP). Invest. Ophthal. Vis. Sci. 37:suppl:3234.

Rajendran, R.R., Van Niel, Gonzalez-Fernandez F (1995) IRBP expression is circadian in zebrafish. Invest. Ophthal. Vis. Sci. 36:suppl:24.

Van Niel, E.E., **Rajendran, R.R.**, Gonzalez-Fernandez F (1995) Teleost interphotoreceptor retinoid binding protein (IRBP). Invest. Ophthal. Vis. Sci. 36:suppl:596.

Rajendran, R.R., Van Niel, E.E, Wagenhorst B.B, Hessler R.B., Gonzalez-Fernandez F (1994) Teleost interphotoreceptor retinoid binding protein (IRBP). Invest. Ophthal. Vis. Sci. 35:suppl:2122.